2. Optimum conditions for the formation of the yellow colour from oestradiol-17β, oestrone and oestriol were almost identical with those of Brown (1955), whereas those for the conversion of yellow into pink differed considerably. The differences are most likely due to the different commercial types of sulphuric acid used in the two methods.

3. Transformation of yellow colour into the pink colour is aided by (*a*) decreasing the sulphuric acid concentration, (*b*) increasing the heating time, (*c*) the presence of oxidizing agents and (*d*) ultraviolet light. Excess of these influences results in fading of the final pink colour.

4. The pink-colour complex once formed under optimum conditions from both pure crystalline compounds and urinary fractions is stable for at least 24 hr. in the absence of bright light.

5. Urinary contaminants reduce the chromogenicity of phenolic steroids. The effects are small and reproducible in a very narrow range when the colour reaction is used in connexion with the Brown purification procedure.

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Biochem. J. (1961) **78**, 602

A New Chromatographic Method for the Determination of Thiamine and its Mono-, Di- and Tri-Phosphates in Animal Tissues

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Some methods hitherto used for the determination of thiamine and its phosphoric acid esters in animal tissues only allow a distinction between free and phosphorylated thiamine (by using thiochrome methods before and after enzymic digestion) (see Association of Vitamin Chemists, 1951), or measurement of cocarboxylase activity (Westenbrink & Steyn-Parvé, 1949; Kaziro, 1957), displayed by thiamine diphosphate alone.

Paper chromatography (Spadoni & Tecce, 1950; Rossi-Fanelli, Siliprandi & Fasella, 1952; Kiessling & Lindahl, 1953; Bartley, 1954; Bernabei & Wildemann, 1959) and paper ionophoresis (Gurtner, 1957) have been widely employed, but usually only

for qualitative assays. On the basis of previous work (de Giuseppe & Rindi, 1958), where we reported the analytical conditions for the separation and determination of thiamine and its phosphoric esters in pure solutions by using a strong anionic resin, we developed a method for the quantitative assay of these compounds in animal tissues. This method is described here.

MATERIAL AND METHODS

Abbreviations. The following are used: TMP, thiamine monophosphate; TDP, thiamine diphosphate; TTP, thiamine triphosphate.

Adsorbent charcoal. This was prepared as described by Siliprandi & Siliprandi (1954) by suspending 10 g. of an activated charcoal (3 SL, produced by Italian C.E.C.A., Milan) in a solution containing 1 g. of cholesteryl stearate (British Drug Houses Ltd.) in 300 ml. of ethyl ether plus 300 ml. of ethanol, and continuously stirring for 48 hr. at room temperature.

After addition of a large excess of water, the charcoal was filtered off through a Büchner funnel and thoroughly washed with water. It was dried in the air and stored in an amber-coloured bottle.

Dowex-1 resin (X8; *acetate form*). This was prepared, from a batch of Dowex-1 (X8; 200–400 mesh) (British Drug Houses Ltd.), as indicated by de Giuseppe & Rindi (1958) and was stored in 0·5M-acetic acid. Before use it was thoroughly washed with water.

Amberlite IRC-50 resin. The resin (British Drug Houses Ltd.) was buffered at pH 4·5, according to the method of Vannatta & Harris (1959).

Cellulose powder. Whatman, ash-free, cellulose powder was washed with 50% (v/v) ethanol, filtered off in a Büchner funnel, thoroughly rinsed with water and dried.

Columns. All the chromatographic columns were made from Pyrex-glass tubes, fitted with a sintered-glass disk, and fused to a ground-glass stopcock. Suitable separatory funnels could be attached to the tubes by means of ground-glass joints.

Flow rate. During all analytical steps (adsorption, washing, elution), the flow rate was 10–15 drops/min.

Preparation of the charcoal columns. A portion (1–2 ml.) of a 10% cellulose suspension in water was placed on the sintered-glass disk of a suitable column. A homogeneous mixture of charcoal and cellulose powder (1:5) was prepared and suspended in water. The suspension was placed on the settled cellulose layer by means of a wide-mouthed pipette, whose tip was kept below the water surface. The charcoal was allowed to sediment completely, by opening the column stopcock, without eliminating all the water. Finally 1 ml. of cellulose suspension in water was placed on the charcoal column after thorough cleaning of the walls by means of a glass rod wadded with cotton.

Determination of thiamine. The Bessey, Lowry & Davis (1952) procedure, which theoretically could have the advantage of avoiding enzyme hydrolysis of the phosphoric esters, was tested, but the thiochrome method (Association of Vitamin Chemists, 1951) was finally used since it gave much more constant results. Since it involves extraction of the thiochrome with *iso*butanol, the thiamine phosphoric esters were hydrolysed by an acid phosphatase before the determination. All the results are expressed as thiamine chloride hydrochloride.

Phosphatase solution. This was prepared, just before use, by suspending 1 g. of Taka-diastase (Parke, Davis and Co. Ltd.) in 10 ml. of 2M-acetate buffer, pH 4·5. Similar results have been obtained by dissolving 0·1 g. of freeze-dried acid phosphatase, prepared from human prostate according to Vescia & Testi (1958) with minor modifications, in 10 ml. of 2M-acetate buffer, pH 5·2.

Apparatus. Beckman DU model G. 4700 spectrophotometer equipped with fluorimetric accessories was used.

RESULTS

Preliminary experiments

In all the experiments described here the direct method for determining thiamine without purification on Decalso (Association of Vitamin Chemists, 1951) was used.

A series of comparative tests carried out on the same trichloroacetic acid extract of rat tissues, before and after treatment with cholesteryl stearate-treated charcoal, gave almost the same results both with extract in the unpurified state and after passage through an Amberlite IRC-50 column, buffered at pH 4·5, according to the method of Vannatta & Harris (1959). In contrast, the use of Decalso led to losses of thiamine up to 50%. The choice of trichloroacetic acid for the extraction was made after comparing the deproteinizing and thiamine-extracting action of various solutions (0·5M-perchloric acid; 5% trichloroacetic acid; 0·1N-hydrochloric acid) on rat liver. Both 5% trichloroacetic acid and 0·5M-perchloric acid had the same extractive power, but the former was preferred, since less protein [estimated by the quantity of Folin–Ciocalteu-positive material (method of Lowry, Rosebrough, Farr & Randall, 1951)] was found in the deproteinized extract. A preliminary purification to remove trichloroacetic acid, salts and the other substances from the extract was necessary for an efficient separation of thiamine and its phosphates on the Dowex column. Siliprandi & Siliprandi (1954) used adsorption on cholesteryl stearate-treated charcoal, followed by elution with 50% ethanol, in order to desalt pure solutions of thiamine phosphates, and obtained excellent recoveries. The same procedure used for rat-tissue extracts gave thiamine recoveries not exceeding 85%.

In an attempt to improve the recoveries, other water-miscible solvents at different concentrations (between 10 and 80%) were tested. Among methanol, ethanol, acetone and propan-1-ol, the best recoveries of extracted and adsorbed thiamine were obtained with 10% propan-1-ol. This solvent is also easily eliminated under vacuum. The stability of thiamine phosphates in trichloroacetic acid was also studied. Solutions of TMP, TDP and

TTP in 5% trichloroacetic acid, containing 3–0·6 μg. of total thiamine/ml., showed almost no splitting of the esters after 5 hr. at 23°.

The use of two Dowex-1 columns for the tissue extract was not as reliable as for pure solution (de Giuseppe & Rindi, 1958). Therefore only one column of Dowex-1 (X8; acetate form) was used; thus TDP and TTP were retained, whereas thiamine and TMP passed down together in the eluate. It was possible to determine thiamine directly by extraction into *iso*butanol and TMP after phosphatase action, because (Westenbrink & Steyn-Parvé, 1949) thiochrome phosphates are not soluble in *iso*butanol, but thiochrome itself is. The difference between total thiamine (after phosphatase) and free thiamine corresponds to TMP. TDP was eluted with 0·02 M-sodium acetate in 0·04 M-acetic acid solution, and TTP by M-acetate buffer, pH 4·5. The eluates were incubated with phosphatase for the determination of total thiamine. For TDP, the determination could be made on the incubation medium, but this was not possible for TTP, because high blanks were obtained. The thiamine liberated from the TTP fraction by enzymic digestion was adsorbed on Amberlite IRC-50 resin, buffered at pH 4·5, and eluted with N-hydrochloric acid. Sufficient purification was achieved so that the blank interference was eliminated.

All the extracts were acidified with 0·1 N-hydrochloric acid before their chromatography on Dowex-1, in order to convert the thiamine and its compounds into hydrochlorides. Unless this was done poor recoveries were obtained.

Recommended procedure

Principle. The tissue was extracted and deproteinized by homogenization in trichloroacetic acid. The extract, brought to pH 6·7–6·8, was adsorbed on a charcoal column and eluted with 10% (v/v) propan-1-ol. The vacuum-concentrated eluate was applied to a column of Dowex-1 (X8; acetate form), which retained TDP and TTP and allowed thiamine and TMP to flow through. The thiamine and TMP content of the percolated fluid and the combined water washings were determined. TDP was eluted from the Dowex column with 0·02 M-sodium acetate in 0·04 M-acetic acid, and TTP by M-acetate buffer, pH 4·5. TDP and TTP were determined in the eluates.

Extraction. The rapidly excised tissue was placed in cold 5% (v/v) trichloroacetic acid, then weighed and homogenized in 2·5 vol. of the extracting fluid with a MSE homogenizer. After centrifuging (3500*g*) at 0° for 5 min., the supernatant fluid was separated and stored at 0°. The residue was again homogenized in 1 vol. of 5% trichloroacetic acid and centrifuged. The combined supernatant fluids were neutralized (pH 6·7–6·8) with 40% (w/v) NaOH. The amount of tissue to be used, which varies with the total thiamine content, is approximately (g.): brain, 13; heart, 5; kidney, 9; liver, 6.

Adsorption on charcoal and elution. The neutralized extract was passed through a layer composed of a mixture of charcoal (300 mg.) and cellulose powder (1·5 g.), placed in a chromatographic column (11 mm. × 111 mm.) as above described. The column was washed with 20–25 ml. of water.

The thiamine compounds were eluted with 60–70 ml. of 10% (v/v) propan-1-ol (Merck, for chromatography) and collected in a round-bottom 500 ml. flask.

Concentration of eluate. The propanol eluate was concentrated to about 5 ml. in a rotating evaporator (Rinco) under vacuum, on a water bath at 25–30°, the pressure being reduced gradually to avoid bumping. Concentration takes about 20 min. The flask was then accurately washed and the volume brought to 15 ml. with water.

Chromatography on Dowex-1. After addition of 0·8 ml. of 0·1 N-HCl, the concentrated eluate was passed through a layer (8 mm. × 25 mm.) of Dowex-1 (X8; acetate form; 200–400 mesh) placed in a chromatographic glass column (8 mm. × 80–90 mm.). The resin was then washed with water, and both eluate and washing were collected in the same 25 ml. volumetric flask, up to the mark, with thorough mixing. Then 0·5 ml. of 2 M-acetate buffer was added to 12·5 ml. of this solution in an Erlenmeyer flask (fraction I), and 0·5 ml. of phosphatase solution was added to the remaining 12·5 ml. (fraction II). About 20 ml. of 0·02 M-sodium acetate in 0·04 M-acetic acid was put on the column. Phosphatase solution (1 ml.) was added to the eluate (fraction III), which contained only TDP, in a 20 ml. volumetric flask, the volume being adjusted to the mark with water if necessary. Finally, TTP was eluted from the resin with about 19 ml. of M-acetate buffer, pH 4·5. The eluate (fraction IV) was treated as indicated for TDP. After thorough mixing, all four fractions were incubated overnight at 37°. Fraction IV, diluted with 20 ml. of water, was then passed through a bed (8 mm. × 40 mm.) of buffered (pH 4·5) Amberlite IRC-50 resin in a glass column (8 mm. × 80 mm.). The resin was washed with 15 ml. of water and eluted with 20 ml. of N-HCl; the eluate was collected in a 20 ml. volumetric flask and the volume was adjusted to the mark (fraction IV A).

Determination of thiamine. The following volumes (ml.) of the fractions were placed in duplicate in two centrifuge tubes (21 mm. × 150 mm.) provided with ground-glass stoppers: I, 5; II, 5; III, 1; IV A, 5. All the volumes were adjusted to 5 ml. with water. A portion (5 ml.) of an aqueous thiamine solution (containing 0·2 μg./ml.) was treated in the same way and served as the standard. Solid potassium chloride (1·25 g.) was placed in each tube, which was then shaken until the solid dissolved.

Both oxidation with ferricyanide and extraction with *iso*butanol (15 ml.) were accomplished according to Association of Vitamin Chemists (1951). For the fluorimetric measurements, the instrument was set with a quinine sulphate solution (0·3 μg./ml.) in 0·1 N-H_2SO_4.

The fluorimetric readings were corrected for the blanks [without oxidation, see Association of Vitamin Chemists (1951)] of fractions I, II, III and IV A, and of the standard. Thiamine is expressed as thiamine chloride hydrochloride, and TMP, TDP and TTP as the compounds themselves.

Recovery of thiamine in the method. The reliability of the method is illustrated by Table 1, which reports the results, expressed as thiamine, of several determinations made on rat tissues, together with recoveries of the various analytical steps.

Table 1. *Recovery of thiamine compounds (expressed as thiamine) from rat tissues during all the analytical steps*

Values given are means±S.E. For details see text. *A*, Crude trichloroacetic extract; *B*, charcoal-purified trichloroacetic extract; TMP, TDP, TTP, the single fractions after Dowex chromatography; *C*, thiamine + TMP + TDP + TTP.

Rat tissue	No. of determinations	Thiamine content (μg./g. of fresh tissue) in fraction				
		A	*B*	*B*/*A* (%)	Thiamine	TMP
Brain	5	2·60±0·09	2·40±0·11	92·1±1·13	0·11±0·01	0·29±0·01
Heart	4	7·28±0·14	6·83±0·28	93·9±2·24	0·15±0·02	0·35±0·02
Kidney	5	4·11±0·01	3·88±0·03	94·2±1·15	0·25±0·01	0·39±0·07
Liver	6	7·25±0·17	6·72±0·14	92·7±0·80	0·24±0·02	0·65±0·03
		TDP	TTP	*C*	*C*/*A* (%)	
Brain	5	2·08±0·13	0·13±0·02	2·62±0·11	100·6±1·33	
Heart	4	6·10±0·39	0·40±0·02	7·00±0·39	96·1±3·54	
Kidney	5	2·87±0·08	0·19±0·01	3·72±0·02	90·3±0·19	
Liver	6	5·40±0·20	0·62±0·09	6·92±0·17	95·4±1·06	

Table 2. *Thiamine and thiamine mono-, di- and tri-phosphate content of some rat tissues*

Values are expressed as μg. of each compound/g. wet wt. of tissue. The values given are means±S.E. The numbers in parentheses are the number of determinations. The percentages show the thiamine content of each fraction as a percentage of the total thiamine of the tissue.

	Brain (5)		Heart (5)	
	(μg./g.)	(%)	(μg./g.)	(%)
Thiamine	0·11±0·08	4·4±0·20	0·16±0·02	2·3±0·35
TMP	0·30±0·09	11·5±0·25	0·41±0·05	5·9±0·91
TDP	2·61±0·15	78·9±1·18	7·44±0·44	86·0±1·06
TTP	0·19±0·03	5·0±0·19	0·57±0·04	5·6±0·48
	Kidney (7)		Liver (6)	
	(μg./g.)	(%)	(μg./g.)	(%)
Thiamine	0·22±0·02	6·2±0·45	0·24±0·01	3·5±0·15
TMP	0·35±0·06	9·8±1·42	0·66±0·01	9·4±0·43
TDP	3·47±0·13	78·6±1·62	6·81±0·44	77·9±1·28
TTP	0·27±0·01	5·2±0·24	0·92±0·13	9·0±1·48

Thiamine and thiamine phosphate content of rat tissues. Table 2 gives the contents of thiamine and its phosphoric esters of some rat tissues, as determined with the present method.

DISCUSSION

The difficulty encountered in determining the content of the various thiamine phosphoric esters in tissues depends on their very low concentration in comparison with the quantity of other substances, particularly nucleotides and salts, which are simultaneously extracted. This necessitates purification of the extract. The use of partially deactivated charcoal, as indicated, is extremely useful for this purpose, because it allows complete desalting, with minimum loss of the thiamine compounds. Considering the small quantity of thiamine compounds carried through the procedure, the average final recovery of 95·4% can be considered satisfactory. With skeletal muscle, whose total thiamine content is very low (1–1·6 μg./g.), we made several determinations of thiamine esters, with a large weight of tissue (20–30 g.) sufficient to contain the suitable amount of total thiamine. Final recoveries (80–85%) were always low because of the large amounts of interfering substances which could not be completely removed by the analytical procedure. Hence we did not consider the present method satisfactory for skeletal muscle. Duplicate determinations agreed within approximately 3%.

The method described improves on various procedures used up to the present, which only partially satisfy the requirements of any determination of thiamine phosphoric esters. The method makes it possible to determine the TMP and TTP content of the tissues. The time needed to perform the whole process is about 12 hr. (excluding the phosphatase incubation period overnight). The results obtained with our method, as regards the total thiamine and TDP content of the various tissues, agree with most of those reported for rat organs in the literature. For example, the total thiamine values collected by Dreyfus (1959) are (μg./g.): brain, 2·2–6·0; heart, 1–7·4; kidney, 3·5–11; liver, 2·8–14; whereas we found respectively 2·6; 7·2; 4·1; 7·2.

For TDP Westenbrink, Goudsmith & Veldman (1941) reported (μg./g.): brain, 3·0; heart, 6·0; kidney, 5·5; liver, 7·0–13·0; and Boffi, Bucci & Lucarelli (1958) for the same tissues: 2·8; 6·6; 4·9; 6·7. In all our determinations, small amounts of TMP and TTP were always found in animal tissues along with thiamine and TDP. Their biochemical significance has still to be discovered. TTP was found in liver by Rossi-Fanelli *et al.* (1952) and in liver, kidney and brain by Greiling & Kiesow (1958). Our observations showed its presence in heart too, but the richest organ analysed was the liver (0·84 μg./g.), followed by the heart (0·59 μg./g.), kidney (0·27 μg./g.) and brain (0·19 μg./g.). The most abundant thiamine compound in rat tissue is TDP, which represents an average of about 80% of the total thiamine present.

SUMMARY

1. A new analytical procedure for the determination of thiamine and its mono-, di- and triphosphoric esters in rat tissue is described.

2. The method allows a mean thiamine recovery of 95%, and good reproducibility.

3. In all the rat tissues studied, thiamine diphosphate was the most abundant thiamine phosphoric ester, but small amounts of thiamine tri- and mono-phosphate were also found.

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Biochem. J. (1961) **78**, 606

The Distribution of Free *meso*Inositol in Mammalian Tissues, Including some Observations on the Lactating Rat

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Since Woolley (1940) first demonstrated that *meso*inositol was an essential dietary factor for the growth of the mouse, there have been several studies on the distribution of total tissue inositol (e.g. Taylor, Pollack & Williams, 1942) and lipid inositol (e.g. Taylor & McKibbin, 1953) in mammals. Surprisingly little attention appears to have been paid to the distribution of free inositol in the tissues, although Woolley (1942) found that 60% of the inositol present in the normal mouse carcass was in the free form, and Platt & Glock (1943) obtained similar results with the rat. Meyer (1946) isolated free crystalline inositol from commercial desiccated thyroid powder, and more recently Kojima & Kusakabe (1954) have obtained it from the brain and kidneys of the pig. Mann (1954) has isolated large amounts of inositol from the seminal vesicular secretion of the boar.

The present paper presents results of assays for free *meso*inositol in the tissues of the rat, guinea pig and rabbit by using the specific microbiological-assay procedure of Campling & Nixon (1954), which can estimate free inositol in the presence of esterified inositol. It shows that the concentration

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